Ribonuclease B of Bovine Milk

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Ribonuclease B has been isolated from 757 liters of milk; pilot plant facilities were used to provide a crude fraction. Final purification was achieved by gel filtration on Sephadex G-75 and chromatography on IRC-50. Milk ribonuclease A was also isolated by this procedure. Milk ribonuclease B has an amino acid composition which is identical to pancreatic ribonuclease A, milk ribonuclease A, and pancreatic ribonuclease B. It is a glycoprotein containing 4.2% hexosamine (3.0% glucosamine plus 1.2% galactosamine) and 5.17% mannose. It differs from pancreatic ribonuclease B, which contains 2.16% glucosamine, no galactosamine, and 5.7% mannose.

Several ribonucleases have been identified in cow's milk. Our previous report (1) presented evidence that the major ribonuclease of bovine milk is identical to pancreatic ribonuclease A. This report describes the isolation and properties of another milk ribonuclease, designated B.

Plummer and Hirs (2, 3) isolated a ribonuclease B from pancreatic juice. The enzyme is a glycoprotein; the carbohydrate is a polysaccharide containing 6 residues of mannose and 2 of glucosamine. The amino acid composition of ribonuclease B is identical to the amino acid composition of ribonuclease A. Their experiments provided evidence that all the carbohydrate is associated with one peptide and is attached to the asparagine residue in position 34. Our studies indicate that milk ribonuclease B differs from pancreatic ribonuclease B in its carbohydrate composition and its chromatographic behavior.

EXPERIMENTAL PROCEDURE

Materials. Pancreatic ribonuclease A (lot No. RASE 6514-15) was purchased from Worthington Biochemical Corporation.[2] As the enzyme was supplied in phosphate buffer, it was desalted on a Sephadex G-25 column, which was equilibrated with 0.1 M acetic acid. This procedure removed 280 mμ absorbing impurities associated with the ribonuclease A, as well as the salt. The final product was homogeneous by disc electrophoresis and by chromatography on IRC-50. Raw skim milk was obtained from a local dairy. Pancreatic ribonuclease B was generously supplied by Dr. T. H. Plummer.

Protein determination. Protein was estimated from the absorbance at 280 mμ. For the pure enzyme, an extinction coefficient of 7.1 was used (4). Protein was also determined by the procedure of Lowry *et al.* (5). When a more sensitive method of determining protein was required, the protein was first hydrolyzed with NaOH and then treated with ninhydrin (6). All absorbance measurements were made in a Beckman spectrophotometer, model DU, using 1-cm quartz cells.

Enzyme activity. Ribonuclease activity was measured by the procedure of Kalnitsky *et al.* (7). Yeast ribonucleic acid (Schwarz Bioresearch, Inc.) was used as a substrate. The ribonucleic acid was adjusted to pH 5 and dialyzed against distilled water for 24 hours before using. This assay was used to follow the fractionation of ribonuclease. Purified yeast ribonucleic acid (Worthington) was used for specific activities on the final ribonuclease preparations.

Disc electrophoresis. The electrophoretic pattern of ribonuclease was examined using disc electrophoresis at pH 4.3 (8).

Amino acid analyses. Samples of 2 mg of protein were hydrolyzed with 6 N HCl for 24 hours at 110° in evacuated sealed tubes. The analyses were performed according to the method of Piez and Morris

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[2] It is not implied that the U.S. Department of Agriculture recommends the above company or its products to the exclusion of others in the same business.

(9). The residue values were determined by assuming 15 residues of aspartic acid per mole of ribonuclease. Corrections were made for the four amino acids which undergo destruction during hydrolysis, using the correction values of Gundlach (10) for serine, threonine, half-cystine, and tyrosine. Analyses were carried out in triplicate.

Carbohydrate analyses. The Dische cysteine-sulfuric acid method (11, 12) was used to estimate hexose, pentose, and methylpentose. Mannose, xylose, and fucose were used as standards. Total reducing sugar was determined by the phenol-sulfuric acid method (13) as modified by Lee and Montgomery (14) with mannose as the standard. The thiobarbituric acid assay of Warren (15) was used for the estimation of sialic acid with *N*-acetyl neuraminic acid as the standard.

The method of Boas (16) was used for the estimation of hexosamine. Protein samples (approximately 3 mg) were hydrolyzed with 2 N HCl for 6 hours at 100° in evacuated sealed tubes. Optimum hydrolysis conditions were established by varying the acid concentration and the time of hydrolysis. The above conditions provided maximum liberation of the hexosamine. The hydrolyzed samples were placed on Dowex-50 columns, washed with water, and eluted with 2 N HCl as described by Boas. The eluted hexosamines were analyzed colorimetrically. Another aliquot was analyzed on the amino acid analyzer according to the method of Piez and Morris (9). By this method glucosamine and galactosamine could be separated. Approximately 0.5 mg of protein was used for each determination.

Constituent hexosamines were also measured on the Technicon amino acid analyzer in an automatic system described by Gregory and Van Lenten (17). Twelve mg of ribonuclease B was hydrolyzed in 3 ml of 3 N HCl in an evacuated sealed tube for 4 hours at 108°, and then evaporated to dryness. The hydrolyzed protein was dissolved in water. Aliquots were added to a column containing Type B Chromobeads and the amino sugars were eluted with borate buffer as described (17). The effluent was analyzed for hexosamine by the Elson-Morgan reaction. The presence of glucosamine and galactosamine was determined by the position of the peak. Mannosamine was used as the internal standard after determining that there was no mannosamine in the ribonuclease B.

The carbohydrate constituents of ribonuclease B were identified by paper chromatography. The procedure described by Plummer and Hirs (2) was followed. Five mg of ribonuclease B was dissolved in 1.0 N HCl and hydrolyzed for 2 hours at 100° in an evacuated sealed tube. The hydrolyzed protein was filtered on a Sephadex G-25 column (0.9 × 85 cm) equilibrated with 0.1 M acetic acid. Aliquots of 0.5 ml were collected. Samples were analyzed by the Lowry procedure for protein and by the phenol-sulfuric acid method for carbohydrate. The carbohydrate-containing fractions were pooled and evaporated to dryness under vacuum. The residue was dissolved in water and used for paper chromatography. The sample was chromatographed on Whatman No. 1 paper, using descending chromatography. Ethyl acetate-pyridine-water (10:4:3) was the solvent employed. Aniline phthalate was used for the detection of carbohydrate. As a confirmatory test for the presence of amino sugars, duplicate paper chromatograms were sprayed with an acetyl-acetone solution, followed by *p*-dimethylamino benzaldehyde reagent as described by Partridge (18).

Column chromatography. Columns of IRC-50 (200–400 mesh) in 0.2 M phosphate buffer were prepared and operated according to the procedure of Hirs *et al.* (19). Some of the columns were operated at high speeds by applying pressure and using finely divided IRC-50 (400–600 mesh) as described by Crestfield *et al.* (20). Crestfield's procedures were followed for analytical column chromatography. Less than a milligram of protein could be analyzed chromatographically in 2 hours using this method.

Gel filtration. Sephadex G-25 and Sephadex G-75 equilibrated with 0.1 M acetic acid were operated according to the procedure of Porath and Flodin (21).

Purification Procedure

The purification procedure that was used previously (1) has been modified. Since the amount of milk was increased 8 times to 757 liters, pilot plant facilities were used to process the milk in the initial stages. Recovery of ribonuclease in the large scale operation was comparable to previous experiments using 95 liters of milk.

Preparation of a crude ribonuclease fraction. 757 liters of raw skim milk was heated to 30° in a 200-gallon tank. The pH of the milk was adjusted to pH 4.6 by the addition of 20 liters of 2 N HCl. The milk was allowed to stand for 2 hours to allow the casein to settle. The casein was removed by filtration through flannel bags.

The pH of the whey was adjusted to 6.2, using approximately 19 liters of 1.5 N NH_4OH. Solid $(NH_4)_2SO_4$ (178 kg) was added to the whey (568 liters) to make the solution 50% saturated. The mixture was filtered by pumping the solution through a plate and frame filter press. "Dicalite" diatomaceous earth (filter aid) was used to coat the filter cloth. The precipitate was discarded.

More solid $(NH_4)_2SO_4$ (146 kg) was added to the filtrate (719 liters) to make the solution 80% saturated. The solution was filtered as above. When

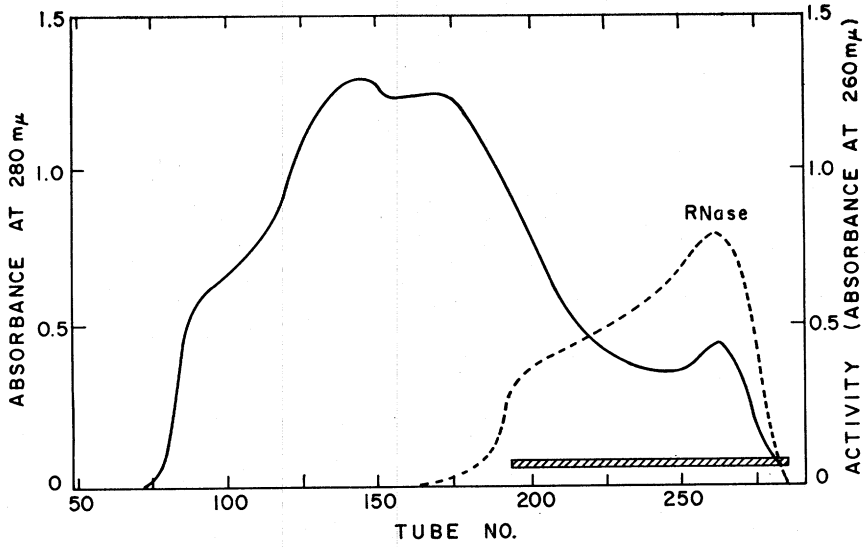

FIG. 1. Chromatography on Sephadex G-75 of proteins eluted from IRC-50. Column measured 9 × 126 cm and was equilibrated with 0.1 M HAc at room temperature. The flow rate was 10 cm/hour and the effluent was collected in 21-ml fractions. The fractions combined for further purification are shown under the bar. The solid line represents protein and the dashed line, ribonuclease activity.

the filtration was completed the filter cloth was spread out and the precipitate was scraped off.

The precipitate was placed in dialysis tubing and dialyzed for 3 days against cold tap water in a 200-gallon tank. The dialyzed solution was filtered through Whatman No. 12 filter paper to remove the filter-aid. The solution was dialyzed for 3 more days against distilled water.

IRC-50 adsorption. IRC-50 in the ammonium form (800 gm) was added to 80 liters of dialyzate and the mixture was stirred for 2 hours. The IRC-50 was allowed to settle and the supernatant solution was discarded. The IRC-50 was washed several times with distilled water. The washed resin was suspended in distilled water and poured into a chromatographic column (9 × 30 cm). Distilled water was allowed to run through the column until the effluent was clear and colorless.

The proteins were then eluted from the column with 1 M ammonium acetate. The proteins emerged as colored bands—orange, green, and red. Ribonuclease was associated with all the proteins, the major portion accompanying the green band. A volume of 2250 ml was eluted containing 41 gm of protein of which 3.6 gm was ribonuclease. The solution was stored at −20°.

Gel filtration. A 250-ml aliquot of the above protein solution was applied to a Sephadex G-75 column (9 × 126 cm). Figure 1 shows the separation of ribonuclease from the major portion of the protein. The ribonuclease was concentrated from the solution (1900 ml) by the addition of solid $(NH_4)_2SO_4$ to 80% saturation. After centrifugation the precipitate was dissolved in water and was refiltered on a Sephadex G-75 column (4 × 60 cm). The fractions containing ribonuclease were combined and lyophilized.

Column chromatography. Ribonuclease A and ribonuclease B were separated on an IRC-50 column, equilibrated with 0.2 M sodium phosphate buffer (pH 6.47), operated according to the procedure of Hirs (19). One gm of protein was dissolved in 0.2 M phosphate buffer (30 ml) and placed on a column (4 × 60 cm). Results of a typical experiment are shown in Fig. 2.

Purification of ribonuclease A. Ribonuclease A (approximately 1200 ml) was placed in a rotary evaporator and the volume was reduced to 150 ml. Salts were removed by passing the solution through a Sephadex G-25 column (4 × 60 cm). The fractions from the protein zone were combined and lyophilized. The ribonuclease A was rechromatographed on IRC-50, pH 6.47, under the same conditions and again freed of salts by gel filtration.

Purification of ribonuclease B. The ribonuclease B was concentrated and freed of salts, using the procedure described for ribonuclease A. The protein was chromatographed on IRC-50 at pH 6.04 (Fig. 3). At this pH, two minor ribonucleases (C and D) were partially separated from ribonuclease B. The ribonuclease B was concentrated, desalted, and rechromatographed under similar conditions.

Figure 4 shows the final purification of ribonuclease B. Total yield was approximately 100 mg. Ribonucleases C and D were not examined further.

RESULTS

The chromatographic properties of milk ribonuclease A, milk ribonuclease B, and pancreatic ribonuclease B are shown in the upper curve of Fig. 5. Since the curves for the two ribonuclease B preparations overlapped, the two proteins were chromatographed together. Figure 5 (lower graph) shows that milk ribonuclease B and pancreatic ribonuclease B can be partially separated chromatographically. Pancreatic ribonuclease A was eluted with the same volume as milk ribonuclease A. When the two ribonuclease A's were chromatographed together, a single peak was observed.

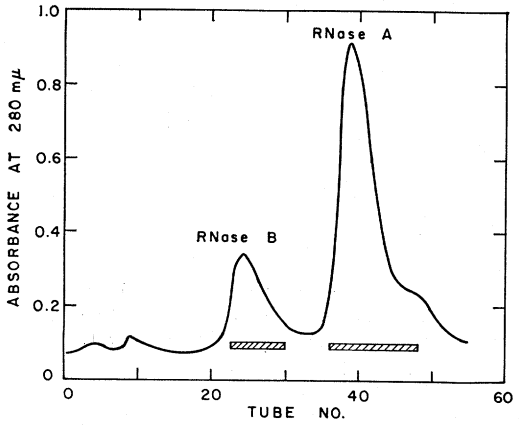

FIG. 2. Separation of ribonucleases A and B on IRC-50. The column measured 4 × 60 cm and was equilibrated with 0.2 M sodium phosphate buffer at pH 6.47 at room temperature. The flow rate was 2.4 cm/hour and the effluent was collected in 22-ml fractions. The fractions combined for further purification are shown by the bar.

Disc electrophoresis at pH 4.3 showed that milk ribonuclease A migrated faster than milk ribonuclease B. There was a noticeable difference in the staining properties. With amido black, ribonuclease A was stained bright blue and ribonuclease B, gray. Milk ribonuclease A and pancreatic ribonuclease A could not be separated electrophoretically. Milk ribonuclease B and pancreatic ribonuclease B could not be separated electrophoretically; however, there was a widening of the band, which suggested that the two proteins could probably be separated under different conditions.

The specific activity of milk ribonuclease B was identical to milk ribonuclease A and pancreatic ribonuclease A, when purified yeast ribonucleic acid was used as the substrate.

The amino acid analyses of milk ribonuclease A and milk ribonuclease B are identical (Table I). The two milk enzymes have

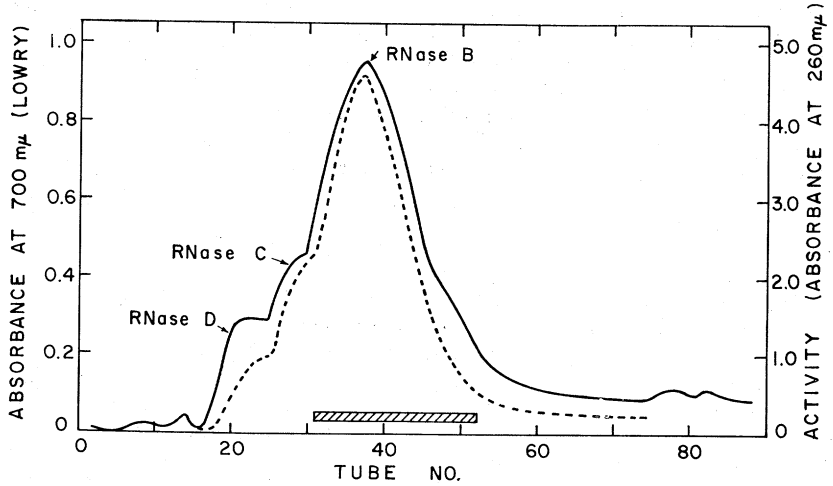

FIG. 3. Chromatography of ribonuclease B on IRC-50. The column measured 3 × 50 cm and was equilibrated with 0.2 M sodium phosphate buffer, pH 6.04, at 4°. The flow rate was 2.35 cm/hour and the effluent was collected in 8.3-ml fractions. The fractions combined for further purification are shown by the bar. The solid line represents protein and the dashed line, ribonuclease activity.

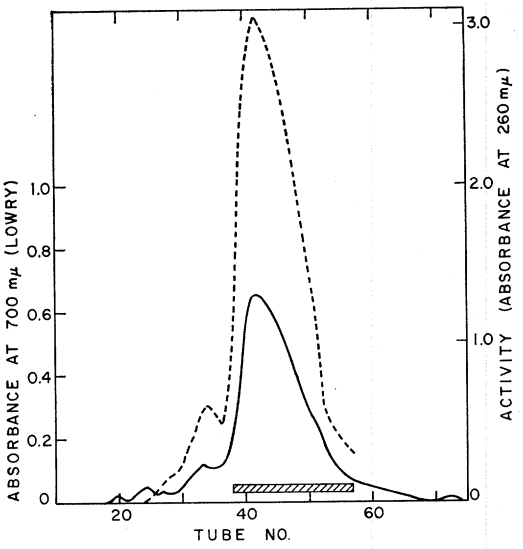

FIG. 4. Rechromatography of ribonuclease B on IRC-50 (400–600 mesh). The column measured 1.9 × 60 cm and was equilibrated with 0.2 M sodium phosphate buffer at pH 6.04 at 4°. Approximately 25 mg of protein was chromatographed. Flow rate was 14.1 cm/hour. The effluent was collected in 4-ml fractions. The solid line represents protein and the dashed line represents ribonuclease activity.

the same amino acid composition as pancreatic ribonuclease A.

Milk ribonuclease B and milk ribonuclease A were tested with the cysteine-sulfuric acid method of Dische (11, 12) for the presence of pentose, methyl pentose, and hexose. Examination of the absorption curves of the reaction products clearly ruled out the presence of pentose and methyl pentose. Ribonuclease B showed the presence of hexose and showed an absorbance curve which was identical to ribonuclease A with added mannose. Sialic acid was not detected with the thiobarbituric assay method of Warren (15).

Paper chromatography was used to identify the carbohydrate constituents. Figure 6 shows that milk ribonuclease B contains mannose, glucosamine, and galactosamine. There was no galactosamine reported for pancreatic ribonuclease B (2).

Values for hexosamines and total reducing sugars are shown in Table II. Milk ribonuclease B contains 5.17% reducing sugars, which is slightly lower than the 5.7% value reported for pancreatic ribonuclease B (2). Total hexosamine content of milk ribonu-

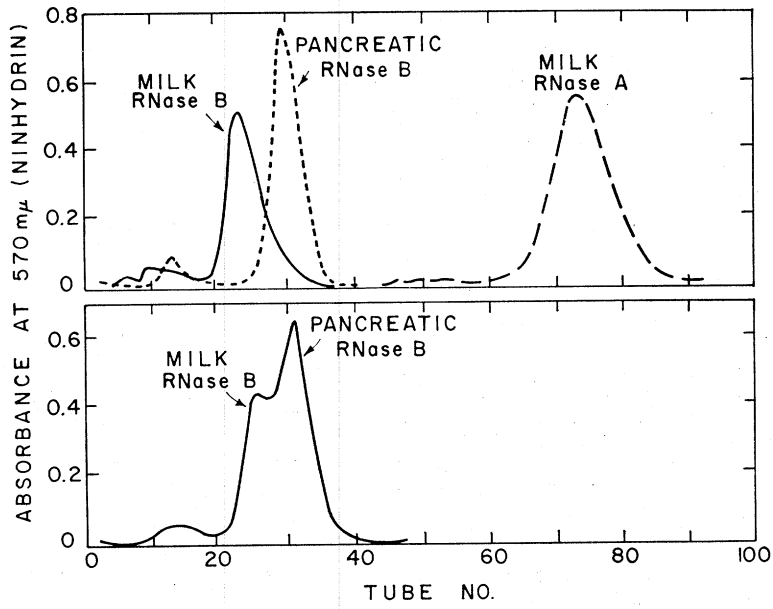

FIG. 5. Analytical chromatography of milk and pancreatic ribonucleases on IRC-50, 400–600 mesh. The flow rate was 52 cm/hour. The column measured 0.9 × 24 cm and was equilibrated with 0.2 M sodium phosphate buffer, pH 6.04. Protein concentration was measured by the ninhydrin method, following alkaline hydrolysis. On the upper curve the three proteins were chromatographed separately. On the lower curve pancreatic ribonuclease B and milk ribonuclease B were chromatographed together.

TABLE I

AMINO ACID COMPOSITION OF MILK RIBONUCLEASE A AND MILK RIBONUCLEASE B COMPARED WITH PANCREATIC RIBONUCLEASE A

Amino acid	Theory	Pancreatic RNase A[a]	Milk RNase A	Milk RNase B
Aspartic acid	15	15.0	15.0	15.0
Threonine	10	9.9	9.9	9.9
Serine	15	14.9	14.9	14.9
Glutamic acid	12	12.1	11.7	11.7
Proline	4	4.2	4.1	4.2
Glycine	3	2.9	3.0	3.3
Alanine	12	12.2	12.1	12.3
Half-cystine	8	7.8	8.9[b]	8.9[b]
Valine	9	8.5	8.8	8.8
Methionine	4	3.8	3.6	3.9
Isoleucine[c]	3	2.2	2.1	2.3
Leucine	2	1.9	2.0	2.1
Tyrosine	6	5.7	6.3	6.1
Phenylalanine	3	2.7	3.0	2.7
Lysine	10	9.7	9.6	9.6
Histidine	4	3.9	4.3	4.3
Arginine	4	3.8	3.5	3.5

[a] Pancreatic ribonuclease A values were taken from the analyses of Riehm and Scheraga (22).

[b] This corrected value for half-cystine is high. Uncorrected value is 7.2.

[c] Isoleucine is known not to be completely liberated in a 24-hour acid hydrolysis.

clease B according to 3 different methods is 4.02%, 4.12%, and 4.6% (See Table II). These values indicate that milk ribonuclease B has approximately twice as much hexosamine as pancreatic ribonuclease B, which has 2.16% hexosamine (2).

Hexosamine was determined on the amino acid analyzer (9). Pancreatic ribonuclease A, pancreatic ribonuclease B, and milk ribonuclease B were compared. Although glucosamine and galactosamine are eluted as separate peaks, glucosamine forms a shoulder on the trailing edge of the glycine peak and galactosamine follows and overlaps cystine. However, a rough estimate of hexosamine can be made by comparing the ribonuclease B pattern with the ribonuclease A pattern and assuming that the amino acid composition is identical. Results confirmed the presence of hexosamine in the ribonuclease B enzymes. Pancreatic ribonuclease B contained 2.0% glucosamine. Milk ribonuclease

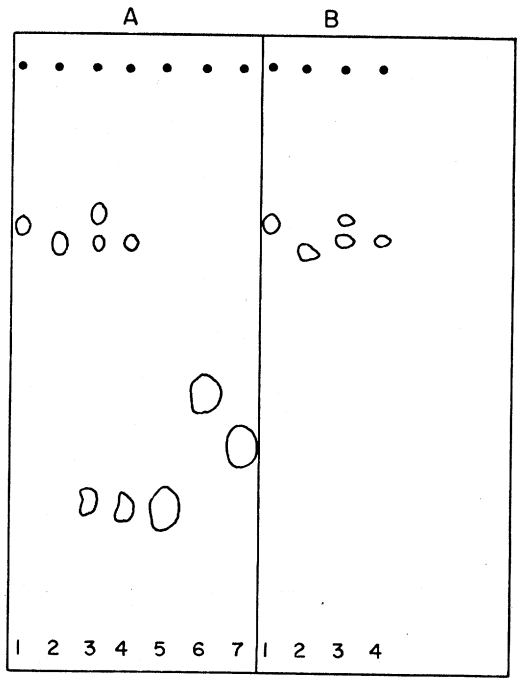

FIG. 6. Descending chromatography with Whatman No. 1 paper. The solvent was ethyl acetate-pyridine-water (10:4:3). Experiment was run for 17 hours. Samples are: 1, galactosamine; 2, glucosamine; 3, ribonuclease B hydrolyzate; 4, milk ribonuclease A hydrolyzate + 5% mannose + 2% glucosamine; 5, mannose; 6, galactose; 7, glucose. A was sprayed with aniline phthalate; B was sprayed with acetyl acetone followed by *p*-dimethylamino benzaldehyde.

TABLE II

CARBOHYDRATE ANALYSES OF MILK RIBONUCLEASE B

Values are expressed as a percentage of the protein. The protein was determined as the absorbance at 280, and an extinction coefficient of 7.1 was used for a 1% solution (4).

Sugar	%	Method used
Total reducing sugar	5.17	Dubois *et al.* (13)
Hexosamine	4.02	Boas (16)
Glucosamine	2.96 } 4.12	Gregory (17)
Galactosamine	1.16 }	Gregory (17)
Glucosamine	3.3 } 4.6	Piez and Morris (9)
Galactosamine	1.3 }	Piez and Morris (9)

B contained 3.3% glucosamine and 1.3% galactosamine (Table II).

Analyses of milk ribonuclease B by the method of Gregory and Van Lenten eliminated the difficulties described in the previous paragraph. Because of the specificity of the Elson-Morgan reaction, amino acids and peptides, if present, were not analyzed and did not interfere. Two well-defined peaks were observed that represented glucosamine and galactosamine. No other amino sugars were observed. Values are 2.96% glucosamine and 1.16% galactosamine, and represent a ratio of glucosamine to galactosamine of 2.55 (see Table II).

DISCUSSION

The occurrence of ribonuclease A in cow's milk and its apparent identity to pancreatic ribonuclease A has been reported (1, 23, 24, 25). The evidence for the identity of the proteins included amino acid analyses, chromatography, immunological behavior, gel electrophoresis, and biological specificity.

A second protein, designated ribonuclease B, has been purified from large quantities of raw skim milk and has been studied in some detail. Two other ribonucleases (C and D) were separated chromatographically but were not investigated further.

Milk ribonuclease B differs from milk ribonuclease A in possessing a carbohydrate moiety; hence it is a glycoprotein. This relationship is similar to that existing between the ribonucleases A and B of pancreatic juice (2, 3). The amino acid compositions of the two proteins are identical (see Table I). Therefore, any differences in physicochemical properties reside in the occurrence in milk ribonuclease B of about 9% carbohydrate, consisting of glucosamine, galactosamine, and mannose. Further, the amino acid compositions of the milk ribonucleases appear to be identical to those of the pancreatic ribonucleases. However, milk ribonuclease B differs in carbohydrate, both quantitatively and qualitatively, from pancreatic ribonuclease B. Milk ribonuclease B has somewhat less reducing sugar (5.2% vs. 5.7%) and more hexosamine (4.0% vs. 2.2%) including galactosamine, which is absent in the pancreatic ribonuclease B. This difference in the carbohydrate content of the milk and pancreatic ribonuclease B is reflected in their differing chromatographic properties, as reported. On the other hand, milk and pancreatic ribonuclease A cannot be distinguished chromatographically under the conditions reported. It has not been determined whether all the carbohydrate of milk ribonuclease B is covalently linked to a single amino acid residue of the polypeptide chain. This is the case in pancreatic ribonuclease B, where the carbohydrate is attached to the asparagine residue in position 34 (3). Although all four ribonucleases appear to have identical amino acid compositions, it cannot be concluded that the protein structures are identical. Complete sequence analyses are needed to establish absolute identity of milk ribonuclease A, milk ribonuclease B, and pancreatic ribonuclease B to pancreatic ribonuclease A.

The site of biosynthesis of the milk ribonucleases is still a matter of speculation. It has previously been suggested that pancreatic ribonuclease A may appear in the milk after intestinal absorption and transport to the mammary gland via the blood stream (1). The same phenomena may account for the occurrence of milk ribonuclease B. However, if the mammary gland and pancreas have independent biosynthetic mechanisms for the ribonucleases isolated, and if these isolated proteins are indeed identical to the proteins synthesized *in vivo*, then the two organs appear to have different mechanisms for producing the carbohydrate portion of the B proteins.

Eylar (26) has suggested that carbohydrate associated with extracellular protein is a label which destines the protein for excretion. The carbohydrate unit, "by interaction with an appropriate receptor or carrier, would act as a chemical passport for exit through the cellular membrane." If this is indeed a fact, then the difference in the carbohydrate composition of milk and pancreatic ribonuclease B might reflect a difference in the interaction of the glycoproteins with the membranes of their respective tissues during the excretory process.

It is of interest that milk has other proteins containing hexose and hexosamine—the red protein (27), κ-casein (28), and the interfacial protein (29). However, these proteins also contain sialic acid.

The isolation of at least two ribonucleases from milk, one containing carbohydrate, would complement the work of Plummer and Hirs (2) with pancreatic ribonucleases and would suggest similar, though not necessarily identical, mechanisms in the synthesis and excretion of these enzymes in two different mammalian tissues.

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